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Experimental pertussis infection in the marmoset: type specificity of active immunity

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SUMMARY

Although we have failed to produce either paroxysmal cough or vomiting in rhesus monkeys, cynomolgus monkeys and marmosets, we have found in marmosets several features of pertussis infection similar to those seen in children with whooping cough: catarrh, persistence of colonization of the naso-pharynx with *Bordetella pertussis* for 4–11 weeks, change of serotype during colonization and inability of type 1 organisms to establish themselves as the predominant serotype.

As in children, we have found that intramuscular vaccine of type 1,2,3 was more effective than type 1,2 in preventing persistent infection with the currently prevalent serotypes 1,2,3 and 1,3. A mixed vaccine (1,2,3 and 1,3) seemed to produce agglutinin 3 in the serum more consistently than a pure type 1,2,3 vaccine. The duration of colonization, after naso-pharyngeal challenge, was greatly reduced in animals with agglutinin 3.

Local immunity, resulting from previous infection, was even more effective than a good vaccine in preventing subsequent persistent colonization. Marmosets may be useful in studying the possible development of aerosol pertussis vaccine for human use.

INTRODUCTION

There is much evidence (see Preston & Stanbridge, 1972) to indicate that the type-specific pertussis agglutinogens (2 and 3) play an important role in immunity to whooping cough in the child. However, in the absence of a field-trial to compare the efficacies of vaccines made from different serotypes of *Bordetella pertussis*, the importance of these antigens is still denied by some workers at international level (Pittman, 1970; Cohen, Hannik & Nagel, 1971).

The experimental animal most widely used in the study of pertussis immunity is the mouse, which can be given a lethal infection by intracerebral injection. Passive type-specific immunity against such infection has been demonstrated by Preston & Evans (1963), but attempts to show active type-specific immunity have failed (Andersen & Bentzon, 1958; Preston & Te Punga, 1959). The most likely explanation (Preston, 1966) is that, in the mouse, the species-specific agglutinogen (factor 1) is more important than the type-specific factors (2 and 3), whereas in man the converse may apply – hence our desire to study type-specific immunity in animals more closely related to man.

Attempts to produce experimental pertussis infection in the respiratory tract of primates range from failure (e.g. Mallory, Horner & Henderson, 1913) to claims that monkeys coughed paroxysmally and sometimes vomited (e.g. Klimenko, 1909; Sauer & Hambrecht, 1929; Lin, 1958; Huang *et al.* 1962). Immunity to re-infection was reported and also successful immunization with pertussis vaccine, but the serotypes of the vaccine strains and challenge strains have not been recorded.

MATERIALS AND METHODS

Strains of Bordetella pertussis

Strain 41633 was isolated in Coventry during the recent whooping-cough survey of the Public Health Laboratory Service (1969). By single colony subculture we isolated, from this one source, pure cultures of the three common serotypes (1,2,3; 1,2; 1,3). All three were lyophilized and they were subsequently used both for attempts to infect marmosets and also for vaccines with which marmosets were immunized.

For intracerebral challenge of vaccinated mice (Kendrick, Eldering, Dixon & Misner, 1947) we used the standard challenge strain, W.18–323, which in our laboratory is a type 1 strain (Preston, 1966).

Serotyping of pertussis strains and vaccines, and estimation of pertussis agglutinins in sera. Full details of these techniques have been described previously (Preston, 1970*b*).

Pertussis vaccines

Growth from charcoal-blood-agar (Oxoid, 1965), incubated at 35–36° C., was harvested in phosphate-buffered saline (pH 7·4) and killed with 0·1% formaldehyde. The bacterial content of each suspension was estimated by comparison with the U.S. National Institutes of Health opacity standard. Marmosets were vaccinated intramuscularly, in the thigh, with four injections over a period of 2 weeks, the total dose for each animal being *ca.* 6×10^{10} bacteria. The hyperimmunized animals (Table 7) received further injections, bringing the total dose to *ca.* 15×10^{10} bacteria.

Marmosets were challenged about 2 weeks after the last injection of vaccine.

Marmosets

The early experiments (Tables 1 and 3) were performed on a number of common marmosets that had been either imported into this country or bred in our Department, but subsequently we used only animals bred in our Department. Their weights ranged from 150 to 350 g., and their ages from 4 months to about 3 years; but we attempted, in each experiment, to ensure that pairs or larger groups were similar in weight-range and sex.

For inoculation in the naso-pharynx, lyophilized strains of *Bord. pertussis* were subcultured three times within a week on charcoal-blood-agar, the third culture was suspended in the basal medium of Cohen & Wheeler (1946), and its bacterial content was estimated by opacity (see above). An inoculum of 0·1 ml., containing

ca. 50×10^9 bacteria, was instilled slowly from a syringe, through a blunted needle, along the floor of the marmoset's nose.

Tiny pernasal swabs, made from brass-wire (*ca.* 0·35 mm. diameter), were used for recovery of *Bordetella* from the naso-pharynx. These were inoculated immediately onto charcoal-blood-agar medium containing penicillin (0·25 units per ml.). For details of culture and identification, see Preston (1970*b*).

Samples of blood (3–4 ml.) were obtained from the femoral vein.

Sedation of primates. Rhesus and cynomolgus monkeys and, for intratracheal inoculation, marmosets were sedated with 'Sernylan' (Parke, Davis and Co., Hounslow, London, England) which was given intramuscularly (1 mg./kg. of body weight) $\frac{1}{2}$–1 hr. before inoculation. Marmosets were inoculated in the naso-pharynx, swabbed and bled without sedation.

RESULTS

Preliminary attempts to infect primates with Bordetella pertussis

Our initial attempts to establish pertussis infection in primates made use of six common marmosets that had been housed in our Department for about 2 years. These were inoculated with two different serotypes of strain 41633 in various doses and by different routes (Table 1), a separate room being used for each serotype. The highest dose, 180×10^9 bacteria, resulted in death 2 days after inoculation. With the other five animals, nothing more than a nasal discharge or catarrh was observed: they did not whoop or vomit. Bouts of coughing did occasionally occur, but only during feeding; and these were observed in normal and inoculated marmosets alike.

Because experimental pertussis infection of rhesus monkeys has been reported previously (Sauer & Hambrecht, 1929) we acquired a young pair, male and female. Both had coughs on arrival, but we did not isolate *Bord. pertussis*, *Bord. parapertussis* or *Bord. bronchiseptica* from either of them, nor were *Bordetella* antibodies detectable in their sera. When they had recovered from their coughs, each was inoculated into the trachea, through the mouth and larynx, with 100×10^9 organisms of strain 41633 (type 1,3). Neither animal showed any ill effect, nor was *Bord. pertussis* recovered with pernasal swabs on any occasion over the next 8 weeks. However, antibodies to *Bord. bronchiseptica* factor *c* (Andersen, 1953) developed in the sera of both animals during this period.

Our failure to infect rhesus monkeys may have been due partly to interference-immunity (Evans & Perkins, 1954) or local antibody resulting from concurrent mild infection with *Bord. bronchiseptica*, but we wondered whether attenuation of the infecting strain of *Bord. pertussis* by lyophilization may have been a factor, and also innate immunity of that type of monkey. We obtained two cynomolgus monkeys, male and female, from the same dealer, and inoculated each of them, partly intratracheally and partly intranasally, with 100×10^9 organisms of a type 1,2,3 strain of *Bord. pertussis* freshly isolated in Manchester. Again there were no signs of illness, and pernasal swabbing failed to recover *Bord. pertussis*. But these

Table 1. *Six non-vaccinated marmosets inoculated in the upper respiratory tract with* Bordetella pertussis, *type* 1,2 *or type* 1,3

Marmoset ...		M3	M5	M1	M2	M4	M6
Inoculum	Route ...	Pernasal	Intratracheal	Intratracheal	Intratracheal	Pernasal	Intratracheal
	Dose ($\times 10^9$ orgs.) ...	30	125	110	55	45	180
	Strain ...	Type 1,2		Type 1,3			
* Serotype(s) isolated with pernasal swabs during *N*th week after inoculation	Week (*N*) 1	1,2	1,2	1,3	1,3	1,3	1,3†
	2	1,2	1,2	1,3	1,3	1,3	—
	3	1,2; 1	1,2	1,3; 1	1,3	1,3	—
	4	1,3; 1	1,2; 1	1,3; 1	1,3; 1	1,3	—
	5	1,3	1,2	1,2; 1,3	1,3; 1	None	—
	6	1,3	1,2; 1	None	None	1,3	—
	7	1,3	None	None	None	None	—
	8–26	None	None	None	None	None	—
Duration of detectable pertussis colonization (days) ...		46	42	30	32	39	2

* This table records only predominant serotypes, and ignores those constituting up to 20% of the colonies tested from the primary plate cultures (see Table 2).

† Died 2 days after inoculation.

Table 2. *Detailed serotyping of* Bordetella pertussis *recovered with pernasal swabs from four marmosets, two inoculated with type* 1,2 *and two with type* 1,3

Marmoset ...		M3				M5				M1				M2			
Inoculum ...		Type 1,2								Type 1,3							
Serotypes of colonies tested ...		1,2,3	1,2	1,3	1	1,2,3	1,2	1,3	1	1,2,3	1,2	1,3	1	1,2,3	1,2	1,3	1
	Week (*N*)																
* No. of colonies of each serotype during *N*th week after inoculation	1	0	18	0	0	0	18	0	0	0	0	12	0	0	0	12	0
	2	0	18	0	0	0	12	0	0	0	0	12	0	0	0	6	0
	3	0	6	0	7	0	12	0	0	1	1	4	2	1	0	11	0
	4	0	0	1	2	2	6	2	4	0	2	11	5	0	0	14	4
	5	0	0	5	0	0	11	1	1	0	1	1	0	0	0	7	3
	6	0	0	18	0	1	5	1	4	0	0	0	0	0	0	0	0
	7	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0

* Each marmoset was swabbed two or three times per week, and about 6 colonies were tested from each primary culture.

animals both had *Bordetella* antibodies in their sera on arrival, and we isolated *Bord. bronchiseptica* type *c* from them on two occasions during the third week after inoculation.

With failure to infect rhesus and cynomolgus monkeys, we reverted to the use of marmosets. Although we had produced no signs of illness except catarrh in the marmoset, we had noted (Tables 1 and 2) several features of pertussis infection that are found in the child (see Preston & Stanbridge, 1972): colonization of the naso-pharynx with *Bord. pertussis* persisted for 4–7 weeks, and change of serotype occurred during colonization; also, type 1 organisms were apparently unable to establish themselves as the predominant serotype, even though they appeared during the course of infection. In order to give ourselves the best chance of detecting signs of pertussis infection, such as coughing, whooping or vomiting, we decided to continue with a large dose (50×10^9 bacteria) which, however, our preliminary study (Table 1) suggested would be sublethal. Also, as there appeared to be no advantage in using intratracheal inoculation, rather than the simpler pernasal procedure, we adopted the latter technique for subsequent experiments.

Relative efficacies of different vaccines against challenge with Bordetella pertussis *of serotypes* 1, 2, 3 *and* 1, 3 (*the types most commonly recovered from children in recent years*)

Against subsequent type 1, 3 challenge (Table 3), vaccines containing antigen 3 had a considerable effect on the duration of colonization. Compared with 36 days for non-vaccinated controls (M13, M31), animals M10, M17 and M11 were colonized for only 8–10 days. Vaccine that did not include antigen 3 appeared to be less effective in eliminating the infection: colonization persisted in M7 and M14 for 20–22 days. (Colonization in M12 lasted as long as in the controls, but this animal had produced no detectable antibody to the factor 3 component of the type 1, 3 vaccine that it received, Table 8.)

The serotypes recovered from animals in which infection persisted for more than 14 days were influenced by the vaccine given previously: infection persisted with a predominance of type 1, 3 in M7 and M14 which had been immunized against antigen 2 (type 1, 2 vaccine); but, in M12, M13 and M31, mutants containing antigen 2 (type 1, 2, 3 and type 1, 2) emerged.

Against subsequent type 1, 2, 3 challenge (Table 4), the influence of the factor 3 content of the vaccine was again apparent. The duration of colonization in M39, M44, M37 and M42 (type 1, 2, 3 or type 1, 3 vaccine) was appreciably less (8–16 days) than in the non-vaccinated controls, M35 and M40 (34–72 days). In all of these animals except M42, the challenge strain mutated from type 1, 2, 3 to type 1, 3 during the course of the infection.

In animals M32 and M43, immunized with type 1, 2 vaccine, these mutants appeared to influence the duration of colonization: in M43 the type 1, 3 mutants did not establish themselves and the infection was eliminated within 14 days, but in M32 a predominance of type 1, 3 developed and the infection persisted for 34 days – as long as in one of the non-vaccinated controls.

These alternative responses to a type 1, 2, 3 challenge after type 1, 2 vaccine were seen again in five more marmosets (Table 5). Rapid elimination occurred, within

Table 3. *Four pairs of marmosets challenged with type* 1,3 Bordetella pertussis *in the naso-pharynx after intramuscular immunization with heterologous and homologous vaccine*

Vaccine ...		Type 1,2,3		Type 1,2		Type 1,3		None	
Marmoset ...		M10	M17	M7	M14	M11	M12	M13	M31
Challenge-strain ...		Type 1,3							
	Week (*N*)								
* Serotype(s) isolated with pernasal swabs during *N*th week after challenge	1	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3
	2	1,3	1,3	1,3	1,3	1,3	1,3	1,2,3; 1,3	1,3
	3	None	None	1,3	1,3	None	1,2,3; 1,3	1,2,3; 1,2; 1,3	1,2,3; 1,3
	4	None	None	None	1,3	None	1,3	1,2; 1,3	1,2,3; 1,2; 1,3
	5	None	None	None	None	None	1,2; 1,3	1,2,3; 1,2	1,2
	6	None	None	None	None	None	1,3	1,2	1,2; 1
	7–9	None	None	None	None	None	None	None	None
Duration of detectable pertussis colonization (days) ...		8	10	20	22	10	36	36	36

* See first footnote to Table 1.

Table 4. *Four pairs of marmosets challenged with type* 1, 2, 3 Bordetella pertussis *in the naso-pharynx after intramuscular immunization with homologous and heterologous vaccine*

Vaccine ...		Type 1, 2, 3		Type 1, 2		Type 1, 3		None	
Marmoset ...		M39	M44	M32	M43	M37	M42	M35	M40
Challenge-strain ...		Type 1, 2, 3							
	Week (*N*)								
* Serotype(s) isolated with pernasal swabs during *N*th week after challenge	1	1, 2, 3; 1, 3	1, 2, 3; 1, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
	2	1, 3	1, 3	1, 3	1, 2, 3; 1, 3	None	1, 2, 3	1, 2, 3; 1, 3	1, 2, 3
	3	1, 3	None†	1, 3	None	1, 3	None	1, 3	1, 2, 3; 1, 3
	4	None	—	1, 3	None	None	None	1, 3	1, 3
	5	None	—	1, 3	None	None	None	1, 3	1, 3
	6	None	—	None	None	None	None	None	1, 3
	7	None	—	None	None	None	None	None	1, 2, 3; 1, 3
	8–10	None	—	None	None	None	None	None	1, 3
	11	NT	—	NT	NT	NT	NT	NT	1, 3
	12–15	NT	—	NT	NT	NT	NT	NT	None
Duration of detectable pertussis colonization (days) ...		16	13	34	13	16	8	34	72

NT = not tested.

* See first footnote to Table 1.

† This animal had apparently eliminated the pertussis infection by the end of the 2nd week; it died during the 3rd week but *Bord. pertussis* was not recovered *post mortem*.

Table 5. *Five more marmosets challenged with type* 1, 2, 3 Bordetella pertussis *in the naso-pharynx after intramuscular immunization with type* 1, 2 *vaccine*

Vaccine ...		Type 1,2				
Marmoset ...		M33	M48	M52	M41	M45
Challenge-strain ...		Type 1,2,3				
	Week (*N*)					
* Serotype(s) isolated with pernasal swabs during *N*th week after challenge	1	1,2,3	1,2,3	1,2,3; 1,3	1,2,3	1,2,3; 1,3
	2	1,2,3; 1,3	1,3	1,3	1,2,3	1,3
	3	1,3	1,3	1,3	None	None
	4	1,3	None	1,3	None	None
	5	1,3	None	1,3	None	None
	6	1,3	None	None	None	None
	7	1,3	1,3	None	None	None
	8	1,3	1,3	None	None	None
	9	1,3	None	None	None	None
	10–12	None	None	None	None	None
Duration of detectable pertussis colonization (days) ...		57	50	32	11	14

* See first footnote to Table 1.

Table 6. *Assessment by the intracerebral mouse-protection test of the relative potencies of the type* 1, 2, 3 *and type* 1, 2 *vaccines used in the immunization of marmosets*

Vaccine (intraperitoneal)		Mortality of mice. No. dead/no. challenged*			PD 50 ($\times 10^8$ orgs.)†
Serotype	Dose ($\times 10^8$ orgs.)	Expt. 1	Expt. 2	Total	
1,2,3	100	1/10	0/10	1/20	14·3
	20	4/10	6/11	10/21	
	4	7/10	9/11	16/21	
1,2	100	0/10	2/11	2/21	9·6
	20	3/8	2/11	5/19	
	4	6/10	9/11	15/21	

* Challenged intracerebrally with *ca.* 150 LD 50 of strain W. 18–323 (type 1).
† Estimated by the method of Reed & Muench (1938).

14 days, in M41 and M45: in M41, type 1, 3 mutants did not replace the parent challenge-strain, and in M45 the type 1, 3 mutants did not become well established but were recovered from the naso-pharynx in only small numbers during the second week. Conversely, in M33, M48 and M52, type 1, 3 mutants did establish themselves and gave rise to a much more persistent infection which lasted for 32–57 days. (The two types of response were *not* related to the sex, age or weight of the animals.)

From Tables 3 and 4 it can be seen that the four animals that were immunized with type 1, 2, 3 vaccine eliminated the subsequent challenge (type 1, 3 or type

1, 2, 3) in 8–16 days (average, 12 days), whereas the four immunized with type 1, 2 vaccine harboured the challenge for 13–34 days (average, 22 days). The greater efficacy of the type 1, 2, 3 vaccine could be explained, as indicated above, on the basis of type-specific immunity, with emphasis on the importance of antigen 3 in these particular experiments. It was also possible, however, that the two vaccines differed in potency in some manner independent of their agglutinogen content, although care had been taken to standardize them to the same opacity. They were therefore compared by the intracerebral mouse-protection test of Kendrick *et al.* (1947) and Table 6 shows that it was the type 1, 2 vaccine which appeared to be slightly more potent by this test, although the difference was not significant.

Efficacies of pertussis vaccines: difficulty in achieving adequate antibody-3 response

The above experiments indicated that the immune response to the factor 3 component of a vaccine may be vitally important in protection against challenge with organisms possessing antigen 3 (type 1, 2, 3 or type 1, 3). Moreover, we noted that one animal (M12) had responded poorly to factor 3 in the type 1, 3 organisms with which it was vaccinated.

We therefore decided to immunize a further series of animals with a higher dose of vaccine (see Materials and Methods) and to compare the responses of groups of marmosets to vaccines of type 1, 2, 3, of type 1, 2 and of type 1, 3 and also to a vaccine consisting of equal parts of type 1, 2, 3 and type 1, 3 organisms.

Table 7 shows an obvious correlation between the titre of agglutinin 3, at the time of challenge, and the duration of colonization with the type 1, 3 challenge strain. Animals in which agglutinin 3 was detectable, even at a low titre (M59, M61, M62, M58, M63, M36, M38) eliminated the challenge-strain in 7–13 days (average, 10 days), whereas animals in which agglutinin 3 was not detectable (M46, M60, M50) harboured the challenge-strain for 18–29 days (average, 23 days). High titres of agglutinins 1 and 2, in these three animals, did not compensate for lack of agglutinin 3.

The absence of agglutinin 3 in M50 was expected, as this animal had received type 1, 2 vaccine. But in M46 and M60 we had further examples of a failure to respond to the factor 3 component of a vaccine, even after a higher dose. We note also that, although the number of animals was too small to draw convincing conclusions, there tended to be a better response to factor 3 in the animals that were vaccinated with a mixture of type 1, 2, 3 and type 1, 3 organisms than in those that received type 1, 2, 3 alone. In both groups, the response to factors 1 and 2 appeared to be more than adequate.

Local immunity from previous infection: better protection than by parenteral vaccine

Table 8 summarizes the results of a first challenge with type 1, 3 or type 1, 2, 3 organisms in thirteen marmosets, some of which had been previously vaccinated. With the exception of animal M43, which has been mentioned previously, there was a correlation between the duration of colonization and the level of serum-agglutinin

Table 7. *Ten marmosets challenged with type* 1,3 Bordetella pertussis *in the naso-pharynx after intramuscular hyperimmunization with various vaccines*

Vaccine ...		Types 1,2,3/1,3			Type 1,2,3				Type 1,2	Type 1,3	
Marmoset ...		M59	M61	M62	M46	M58	M60	M63	M50	M36	M38
Serum-agglutinin titres when challenged	Factor 1	160	320	320	80	1280	1280	320	160	80	80
	Factor 2	640	1280	>1280	1280	>1280	1280	1280	>2560	<5	<5
	Factor 3	40	5	40	<5	10	<5	5	<5	320	320
Challenge-strain ...		Type 1,3									
	Week (*N*)										
* Serotype(s) isolated with pernasal swabs during *N*th week after challenge	1	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3
	2	1,3	1,3	1,3	1,3	1,3	1,3	None	1,3	1,3	1,3
	3	None	None	None	1,3	None	1,3	None	1,3	None	None
	4	None	None	None	1,3	None	1,3	None	None	None	None
	5	None	None	None	1,3	None	None	None	None	None	None
	6–8	None	None	None	None	None	None	None	None	None	None
Duration of detectable pertussis colonization (days) ...		10	10	8	29	13	23	7	18	10	13

* See first footnote to Table 1.

Table 8. *Thirteen marmosets re-challenged with* Bordetella pertussis *in the naso-pharynx after elimination of previous challenge*

Vaccine ...		Type 1,2,3		Type 1,2		Type 1,3		None		Type 1,2		Type 1,3	None	
Marmoset ...		M10	M17	M7	M14	M11	M12	M13	M31	M32	M43	M42	M35	M40
Serum-agglutinin titres at first challenge	Factor 1	160	40	20	20	40	40	<10	<5	10	20	20	<5	<5
	Factor 2	20	160	<10	<10	<10	<10	<10	<5	20	5	<5	<5	<5
	Factor 3	20	10	<10	<10	10	<10	<10	<5	<5	<5	5	<5	<5
First challenge ...		Type 1,3								Type 1,2,3				
Duration of colonization (days) ...		8	10	20	22	10	36	36	36	34	13	8	34	72
Antigens detected from pernasal swabs ...		1:3		1:2:3		1:3	1:2:3			1:2:3				
Serum-agglutinin titres at second challenge	Factor 1	80	160	40	160	320	80	20	<5	40	40	40	<2	<2
	Factor 2	40	320	40	40	<10	<5	<5	<5	160	40	<5	<2	<2
	Factor 3	80	40	<5	<5	40	40	<5	20	<5	<5	80	<2	<2
Second challenge ...		Type 1,2,3								Type 1,3				
*Duration of colonization (days) ...		1	1	3	2	1	2	1	10	4	4	3	4	4
Antigens detected from pernasal swabs ...		1:2:3								(All 3 previously detected)				
Serum-agglutinin titres at third challenge	Factor 1	Not tested					80	80	<5	Not tested				
	Factor 2						<5	<5	<5					
	Factor 3						80	<5	40					
Third challenge ...							Type 1,3							
*Duration of colonization (days) ...							7	2	4					

* After second and third challenge, pernasal swabbing was continued for three weeks (and longer with some animals); the serotype that was recovered, during the few days that colonization persisted, remained the same as that of the challenge-strain.

Table 9. *Comparative efficacy of different vaccines in enabling marmosets to eliminate* Bordetella pertussis *from the naso-pharynx after challenge with organisms possessing antigen* 3 (*type* 1,2,3 *or type* 1,3)*

Vaccine ...	Types 1,2,3/1,3	Type 1,2,3	Type 1,2	Type 1,3	None
No. of marmosets challenged ...	3	8	10	6	4
No. in which colonization persisted less than 14 days ...	3	5	2	4	0
Percentage ...	100	63	20	67	0

* Data from Tables 3, 4, 5 and 7.

for factor 3: those animals with detectable agglutinin 3 eliminated the challenge within 14 days, whereas it persisted much longer in the others.

During the period of colonization, organisms possessing antigens 1 and 3 were recovered from all 13 animals and antigen 2 was detected (either from the original challenge strain or by mutation from a type 1,3 challenge) in organisms recovered from all but three of the animals (M10, M17, M11). But detectable serum-agglutinins did not always develop in the absence of previous vaccination. In particular, animals M35 and M40 produced no detectable serum-agglutinin for any of the three pertussis factors. Nevertheless, on second challenge, even with a strain heterologous to that used for the first challenge, there was very rapid elimination in 1–10 days (average, 3 days). This suggests that the local immunity resulting from previous infection may be much more effective than even a good vaccine in preventing subsequent persistent colonization.

DISCUSSION

The efficacy of intramuscular pertussis vaccine, in hastening the elimination of *Bord. pertussis* subsequently instilled into the nasopharynx of marmosets, has been shown to depend on the level of serum-agglutinins produced in response to the vaccine (Tables 7 and 8). The challenge-strains used in these experiments were of type 1,2,3 or type 1,3 – the types most commonly recovered from children with whooping cough in many countries over the last 10 years (see Preston, 1970*a*; Preston & Stanbridge, 1972; Shmilovitz, Preston, Zaltser & Cahana, 1972; Public Health Laboratory Service, 1973). Since the challenge always included antigen 3, it is not surprising that the level of agglutinin 3 was found to be of great importance, and to this extent the active immunity after vaccination was found to be type-specific – high levels of agglutinins 1 and 2 did not compensate for lack of agglutinin 3. Thus Table 5 shows the limited efficacy of type 1,2 vaccine in protecting against type 1,2,3 infection: as expected, none of these five marmosets had detectable agglutinin 3 when challenged, and prolonged colonization depended on chance mutation of the challenge strain to type 1,3 and early proliferation of this mutant before the original type 1,2,3 challenge could be eliminated with the aid of agglutinins 1 and 2 alone.

Type 1,3 strains possess predominantly antigen 3, with little antigen 1 (Preston, 1971), whereas type 1,2 and type 1,2,3 strains have a high content of antigen 1 and of antigen 2. Immunity to all three serotypes may therefore be achieved by agglutinin 3 together with either agglutinin 1 or agglutinin 2. This would explain why Pillemer's SPA vaccine (Pillemer, Blum & Lepow, 1954), which produced a good antibody-response to factors 1 and 3 (Preston & Te Punga, 1959), gave good protection against whooping cough (Medical Research Council, 1959) at a time when the predominant serotypes in the population were 1,2,3 and 1,2 (Preston, 1963).

In many animals, mutants of the challenge strain proliferated during the course of infection – for example in M1 (Table 1) type 1,3 mutated via type 1 to type 1,2; in M3 (Table 1) type 1,2 mutated via type 1 to type 1,3; in M12, M13 and M31 (Table 3) type 1,3 mutated via type 1,2,3 to type 1,2; in M5 (Table 2) type 1,2 probably mutated directly to type 1,2,3 and also mutated, via either type 1,2,3 or type 1, to type 1,3; and in many animals (Tables 4 and 5) type 1,2,3 mutated to type 1,3. In many of these cases, the selection (or lack of selection) of the mutant was clearly determined by the vaccination history of the animal. This would adequately explain the otherwise rather puzzling finding of the Public Health Laboratory Service (1973) that in 22 of 158 households the serotypes isolated from different children in one house were not the same.

The superiority of type 1,2,3 vaccine over type 1,2 vaccine (Tables 3 and 4) could be explained by the improved ability of these vaccinated animals to eliminate a type 1,3 challenge or type 1,3 mutants from a type 1,2,3 challenge. For the best immunity, vaccine should stimulate the production of all three agglutinins. As in children (Abbott, Preston & Mackay, 1971) it seems that the response to factor 3 is the most difficult to achieve; and Table 7 shows that, when the factor 2 component was reduced by mixing type 1,2,3 organisms with type 1,3 in equal parts, the response to factor 3 was better than with type 1,2,3 organisms alone. Table 9 shows the relative efficacies of vaccines with various combinations of the three pertussis agglutinogens, and suggests that the mixed 1,2,3/1,3 vaccine may well give the best immunity. The criterion, of colonization persisting for less than 14 days from challenge, has been chosen on the grounds that, if a child were colonized for less than 14 days, it would have reached only the catarrhal phase of pertussis infection before the organisms were eliminated, and this would probably result in only mild illness, not recognizable clinically as whooping cough.

Lastly, it is clear from Table 8 that the local immunity resulting from previous infection of marmosets was usually more effective than the best serum-agglutinin immunity achieved by intramuscular vaccine, and this confirms the findings of Huang *et al.* (1962) who showed that immunity to re-infection did not depend on detectable serum-agglutinin. Holt (1972) has also suggested the importance of local immunity and the possibility of introducing aerosol vaccination for children. We would note that nothing is yet known about the type-specificity of such local immunity. After colonization with *any* live serotype, local IgA is likely to include antibody to all three pertussis factors, because of mutations. However, with mutation occurring so readily *in vivo*, it is unlikely that a safe, live, attenuated

vaccine could be developed in the case of *Bord. pertussis*. Before an aerosol vaccine could be introduced, it would be necessary to study the type-specificity and the likely duration of such immunity resulting from locally administered *killed* vaccine, and it appears that the marmoset would be an excellent experimental animal for such studies.

Meanwhile we stress once again (see Preston & Stanbridge, 1972) the importance of an adequate response to the agglutinogen 3 component of parenteral vaccine.

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